Attachment: TCE Developmental Cardiac Toxicity Assessment Update

Introduction

This document is an update on the potential for cardiac defects resulting from exposures to trichloroethylene (TCE), CAS No. 79-01-6.

Background: TCE is a volatile chemical and widely used chlorinated solvent. It is one of the most common man-made chemicals found in the environment. TCE is frequently found in soil and ground water at Superfund sites across the country, and its movement from contaminated ground water and soil into the indoor air of overlying buildings (i.e., vapor intrusion) is of concern.
EPA completed an IRIS assessment of trichloroethylene (TCE) in September 2011 U.S. EPA (2011). After the IRIS document was finalized, some concerns were raised with respect to short-term exposures to TCE and one of the health effects, fetal heart defects, identified in the IRIS assessment and on which the inhalation reference concentration is partially based. A study by Johnson et al. (2003), which reports the results of research on TCE in drinking water, including the findings of Dawson et al. (1993), is included in a group of studies on which the reference values are based in the 2011 IRIS assessment, and is one of several lines of evidence regarding the potential developmental toxicity of TCE. Concerns have been raised by stakeholders about the Johnson et al. (2003) study and EPA's use of this study for short-term risk evaluation. Specific issues raised include the need for 1) a systematic evaluation of study quality, 2) a detailed description of the study design (e.g., the source of concurrent controls), 3) a reexamination of the dose response for cardiac defects, and 4) an evaluation of the study results in light of studies that did not observe cardiac defects with in utero exposures. In addition, concerns have been raised regarding the interpretation of the epidemiological database for cardiac defects associated with TCE exposures.

Purpose: To address the identified issues and to ensure rigorous scientific review of associations between short-term exposure to TCE and fetal cardiac defects, EPA decided to update the analysis of the developmental cardiac toxicity data.

Scope: This report covers only the fetal cardiac defects observed following gestational exposures to TCE and/or its oxidative metabolites dichloroacetic acid (DCA) and trichloroacetic acid (TCA), and does not provide an update on other developmental effects of TCE exposure, i.e., ocular malformations, developmental neurotoxicity, and developmental immunotoxicity. This update includes 1) identification of any new literature, 2) a systematic evaluation of available data, 3) an evaluation of the weight of evidence for the association of TCE exposures with cardiac defects, 4) a reexamination of the dose-response for cardiac defects, and 5) transparent presentation of the evaluation. This process is aligned with the NRC (2011) recommendation for systematic review and weight of evidence evaluation, and presentation of information to increase transparency. A multi-disciplinary team of EPA scientists with expertise in developmental toxicology or biology, epidemiology, statistics, molecular biology, risk assessment, and or TCE toxicology was assembled from across ORD to conduct this assessment.

Literature Search Update

A systematic literature search was conducted to identify all studies published subsequent to the final literature search that had been conducted by EPA during completion of the 2011 IRIS assessment (U.S. EPA, 2011). A total of 1686 unique citations were initially identified from PubMed, Toxline, and Web of Science (WoS). These citations were screened using the title, abstract, and/or full text for pertinence to evaluation of the developmental toxicity of TCE, TCA, and DCA exposure. The literature search identified no new animal toxicology studies of fetal cardiac defects, one new epidemiology study that assessed the association of TCE or chlorinated solvent exposures with cardiac defects, and two studies that provided mechanistic information relevant to alterations of cardiac development following TCE (or metabolite) exposures.

Study Quality Review

For each epidemiology and toxicology study in the developmental toxicity database for TCE, whether previously identified or newly identified in the updated literature search, a detailed review of study quality was conducted.

- **Epidemiology data:** Study quality evaluation criteria and a general format for the capture of epidemiology study data and characterization have previously been developed in IRIS and have been presented in the *Guidelines for Developmental Toxicity Risk Assessment* (U.S. EPA, 1991). These factors include the study power, potential bias in data collection, selection bias, measurement biases associated with exposure and outcome, and consideration of potential confounding and effect modification. This format was used by a team epidemiologist to summarize study information and observed strengths, biases, and confounding factors for each study. An independent review of the conclusions was conducted by the IRIS Epidemiology Disciplinary Workgroup.
- **Animal toxicology data:** Study quality evaluation criteria for in vivo, in vitro, and in ovo developmental toxicology studies were developed. These criteria included considerations described in U.S. EPA (1991) and focused on the adequacy of study design and documentation of information on the test subjects (e.g., species, strain, source, sex, age/lifestage/embryonic stage), environment (e.g., husbandry, culture medium), test substance (e.g., identification, purity, analytical confirmation of stability and concentration), treatment (e.g., dose levels, controls, vehicle, group sizes, duration, route of administration), endpoints evaluated (e.g., schedule of evaluation, randomization and blinding procedures, assessment methods), and reporting (quality and completeness). An independent assessment of each in vivo mammalian study was conducted by at least 2 separate team members, and all team members evaluated study quality for 4 in vivo studies that included a detailed evaluation of cardiac defects (Carney et al., 2006; Johnson et al., 2003; Fisher et al., 2001; Dawson et al., 1993). Individual analyses of study strengths and limitations were collated into a summary table.

Characterization of developmental (cardiac defect) outcomes

Epidemiology Data

Six epidemiologic studies are available on cardiac defects and TCE exposure (Ruckart et al., 2013; Forand et al., 2012; Yauck et al., 2004; Bove, 1996; Bove et al., 1995; Goldberg et al., 1990; Lagakos et al., 1986); five of the six studies were reviewed in the EPA's 2011 Trichloroethylene Toxicological Review (U.S. EPA, 2011). All studies examined oral exposure except Forand et al. (2012) and Yauck et al. (2004) who examined the inhalation exposure route. Forand et al. (2012) is the publication of (ATSDR (2008); 2006) reports referenced in U.S. EPA (2011). Bove (1996) and Bove et al. (1995) report on the same subjects and identical work. (Ruckart et al., 2013), a study published after U.S. EPA (2011), did not draw any conclusions concerning TCE exposure and the occurrence of all cardiac defects or conotruncal heart defects and, for this reason, is not discussed below.

Both Forand et al. (2012) and Bove (1996)/Bove et al. (1995) provide evidence for an association between maternal exposure to TCE or TCE and other chlorinated solvents in drinking water and cardiac

defects. There were differences of opinions among the epidemiology team on Bove (1996)/Bove et al. (1995) due to the few observed TCE-exposed cardiac defect cases, sparse reporting on TCE exposure and cardiac defects in both publications, and the study's cross-sectional design that could not establish temporality. Two other studies with greater potential for biases observed elevated risk estimates between TCE exposure and cardiac defects (Yauck et al., 2004; Goldberg et al., 1990) and support observations in Forand et al. (2012) and Bove (1996)/Bove et al. (1995). There was no evidence of an association between receiving TCE–contaminated water and cardiac defects in Lagakos et al. (1986). The finding of an association between TCE exposure and cardiac defects has coherence with the broader epidemiologic literature that observed association between maternal occupational exposure to degreasing solvents or to organic solvents and cardiac defects (Gilboa et al., 2012; Loffredo et al., 1991; Tikkanen and Heinonen, 1991; Tikkanen and Heinonen, 1988). Although associations are observed in several studies, overall, the studies could not establish that the association on TCE exposure and cardiac defects was causal.
Studies were of different populations, living in different states, and of different epidemiologic designs. Forand et al. (2012) is a small retrospective cohort study of 1,440 live births among New York residents in a TCE contaminated area via vapor intrusion. Bove (1996)/Bove et al. (1995), a cross-sectional study on residents in Northern New Jersey receiving TCE in municipal water supplies, was of 80,938 singleton live-born infants and 594 singleton fetal deaths. A strength of both studies is the use of state record bases, including State Birth Defects Registry with medically-verified outcomes that will reduce information and subject recall bias, and control for potential confounding factors. Both of the studies observed an elevated risk estimate for major cardiac defects: a risk of 1.24 (50% confidence interval (CI): 0.75, 1.94) for >10 ppb TCE in municipal drinking water supplies compared to TCE exposure $\leq$1 ppb and an estimated risk of 2.40 (95% CI: 1.00, 5.77) compared to the rest of New York State, excluding New York City in Bove (1996)/(Bove et al., 1995) and Forand et al. (2012), respectively. Both studies report risk estimates for specific defects: 1.30 (50% CI: 0.88, 1.87) for ventricular septal defects and exposure to >5 ppb TCE in drinking water compared to no exposure Bove (1996)/Bove et al. (1995) and 4.91 (95% CI: 1.58, 15.24) for conotruncal defect compared to no exposure (Forand et al., 2012). Yauck et al. (2004), a small case-control study of 245 cases and 3,780 controls, living within 1.32 miles from at least one TCE emissions source, which used an insensitive exposure surrogate, observed a strong relative risk estimate of 6.2 (95% CI: 2.6, 14.5) for cardiac defects in infants born to mothers aged 38 years or older after controlling for potential confounding. No association for cardiac defects was observed among infants of mothers aged less than 38 years (RR = 0.9, 95% CI: 0.6, 1.2). The case-control study by Goldberg et al. (1990) used three sets of controls, two of which are subject to potential selection bias, and is limited in reporting. Comparing offspring of residentially-exposed mother to infant of non–exposed family, the control group believed to have a less potential for selection bias, an unadjusted risk estimate of 2.58 (95% CI: 2.0, 3.4) was reported by Bove et al. (2002).
Neither Forand et al. (2012) nor Bove (1996)/Bove et al. (1995) present exposure-response information, and the only two studies that do present exposure-response information, do not observe an exposure-response relationship (Goldberg et al., 1990; Lagakos et al., 1986).
In summary, Forand et al. (2012) and Bove (1996)/Bove et al. (1995) provide evidence for association between maternal TCE exposure and cardiac defects. A more mixed pattern of associations is seen in three other studies with greater potential for bias and confounding (Yauck et al., 2004; Goldberg et al., 1990; Lagakos et al., 1986); but, the association in these studies is not considered inconsistent with Forand et al. (2012) or Bove (1996)/Bove et al. (1995). For the database as a whole, the epidemiologic studies are of lower statistical power due to the rarity of cardiac defects. Additionally, information bias of exposure in all studies may provide alternative explanations. As exposure assessment in these studies is at an aggregate level, one can assume information bias is non-differential. Non-differential misclassification of exposure would introduce imprecision, resulting in wider confidence intervals inhibiting the ability to detect some associations and possibly exposure-response relationships. This may be an explanation for the lack of statistically significant findings at the 95% confidence level in Bove

(1996)/Bove et al. (1995). None of the studies considers maternal folic acid intake, a risk factor for cardiac defects. It is unclear whether maternal folic acid intake is a confounder of the associations that were noted since there is no available information to evaluate whether folic acid intake differs related to exposure. However, both Forand et al. (2012) and Bove (1996)/Bove et al. (1995) adjust or examine maternal risk factors, including adequate prenatal care as potential confounding factors. Observations in the other studies are more uncertain compared to Forand et al. (2012) and Bove (1996)/Bove et al. (1995), and may be due to alternative explanations from bias, chance, or potential confounding. Use of hospital cases by Yauck et al. (2004) and cases identified from cardiologist's records by Goldberg et al. (1990) may introduce possible selection bias. It is difficult to evaluate control for potential confounding in Goldberg et al. (1990) due to limited reporting in the publication. The self-reporting of outcome in Lagakos et al. (1986) will introduce uncertainty because of potential selective reporting.

Animal Toxicology Data

The experimental toxicology database for the assessment of developmental cardiac defects resulting from TCE exposures includes in ovo chicken studies, in vitro assays, and rodent studies that assessed fetal morphology following in utero exposures to TCE or its oxidative metabolites.

Inhalation rodent and rabbit TCE studies: Five publications reported the conduct of studies in which TCE was administered by inhalation exposure to rats, using a prenatal developmental toxicity study design (Carney et al., 2006; Healy et al., 1982; Hardin et al., 1981; Dorfmueller et al., 1979; Schwetz et al., 1975). The studies by Hardin et al. (1981) also exposed rabbits to TCE, and the study by Schwetz et al. (1975) also exposed mice. None of these studies reported cardiac defects in fetuses following in utero exposures to TCE; however, of these, only the Carney et al. (2006) and Schwetz et al. (1975) provided sufficient study detail to demonstrate that they were conducted in accordance with good laboratory practices and examined the fetuses using methods designed to detect abnormalities of cardiac development.

Oral rodent TCE studies: Six studies reported the results of oral administration of TCE to rodents during fetal developmental (Johnson et al., 2003; Fisher et al., 2001; Narotsky and Kavlock, 1995; Narotsky et al., 1995; Dawson et al., 1993; Cosby and Dukelow, 1992). All studies were performed in rats, except Cosby and Dukelow (1992) which used mice. In all of these rodent studies, TCE was administered by gavage, with the exception of the Dawson et al. (1993) and Johnson et al. (2003) studies, in which TCE was administered via drinking water. Only the drinking water studies detected treatment-related fetal cardiac defects.

The gavage studies by Fisher et al. (2001), Narotsky et al. (1995), and Narotsky and Kavlock (1995) were conducted in accordance with good laboratory procedures. Fisher et al. (2001) used the same fetal evaluation methods as described in Johnson et al. (2003), and the first author of the Johnson et al. (2003) paper participated in the Fisher et al. (2001) study, yet TCE-related cardiac defects were not detected. The studies by Narotsky et al. (1995) and Narotsky and Kavlock (1995) evaluated neonatal growth and viability, and examined cardiac and other soft tissue morphology only in pups that had died; no cardiac defects were reported. The study by Cosby and Dukelow (1992) did not conduct a detailed assessment of cardiac development.

The Johnson et al. (2003) publication reported the results of TCE drinking water exposures on fetal cardiac development from a 6-year academic research program. It included data on two TCE treatment groups conducted in 1989-1991 that had previously been published by Dawson et al. (1993), plus the data from two lower dose TCE treatment groups conducted in 1994-1995. Cardiac malformation incidence data were compared between treated groups and combined control data from cohorts conducted concurrent to treated groups during the 6-year research program, including controls from studies on TCE metabolites, published in Johnson et al. (1998). Other information on the TCE studies reported in Johnson et al. (2003) included published communications (Hardin et al., 2004; Johnson et al., 2004), errata (Johnson et al., 2005)), and individual cardiac malformation findings and evaluation methods provided to EPA by the study author (Johnson, personal communications, 2009, 2014). Johnson et al. (2003) summarized the combined results from the studies that administered TCE to pregnant rats at doses of 2.5 ppb, 250 ppb, 1.5 ppm, and 1100 ppm in drinking water throughout gestation. Fetal cardiac defects, primarily valvular and septal anomalies, were observed at ≥250 ppb. The 2011 IRIS assessment noted that there are limitations in the Johnson et al. (2003) study. The current evaluation of the Johnson et al. (2003) and Dawson et al. (1993) studies reaffirmed this judgment; study design and reporting issues were identified. The ORD TCE team contacted the study author (P. Johnson), who provided clarification on a number of topics, i.e., a detailed description of study methods beyond what has been previously published, including verification that concurrent controls were conducted for each of the treated groups, information on fetal randomization and blinded cardiac evaluation procedures, and details of animal care and maintenance. As a result of discussions with the study author, an errata was submitted to *Environmental Health Perspectives* (Johnson et al., 2014) to update the public record regarding Johnson et al. (2003). However, some questions on that study remain unresolved, i.e., the precise dates that each individual control animal was on study and the detailed results of analytical chemistry testing for dose concentration. Additional possible sources of uncertainty identified for these studies include that the research was conducted over a 6-year period, combined control data were used for comparison to treated groups, and possible imprecision of exposure characterization due to the use of tap water in the Dawson et al. (1993) study and TCE intake values that were derived from water consumption measures of group housed animals. On the other hand, the strengths of this study include the examination of fetal hearts without knowledge of treatment (or control) group, standardized methods of fetal evaluation, examination of the gross (in situ) and internal structure of the fetal hearts by a group of 3 senior researchers, confirmation of cardiac anomalies by consensus agreement, and that the researchers shared individual fetal and litter cardiac abnormality data for treated groups with EPA, thereby facilitating independent statistical analysis of the data.

Oral rodent metabolite studies: Several studies were conducted in rats to examine the effects of developmental exposures to the TCE oxidative metabolites, DCA and TCA. Studies by Smith et al. (1992) and Epstein et al. (1992) observed cardiac defects following gavage administration of DCA during pregnancy. Smith et al. (1989) and Johnson et al. (1998) reported cardiac defects with TCA exposures administered during gestation via gavage and drinking water, respectively. However, a well-conducted study by Fisher et al. (2001) did not detect cardiac defects following gavage administration of DCA or TCA on GD 6-15.

In ovo avian studies: Several studies examined cardiac development following in ovo administration of TCE to chicken embryos (Rufer et al., 2010; Drake et al., 2006b; Drake et al., 2006a; Loeber et al., 1988). Abnormalities of cardiac structure and/or function were observed in each of these studies. Defects in valvuloseptal development were similar to those that have been observed in rodents and humans, since early stages of cardiac development are similar across species (NRC, 2006).

In vitro assays: Whole embryo culture studies that examined cardiac development were conducted by Hunter et al. (1996) using mouse embryos exposed to DCA or TCA and by Mishima et al. (2006) using chicken embryos exposed to TCE. Alterations in cardiac development were observed in each of these models.

Summary of animal toxicology data: Alterations in fetal cardiac development have been observed in rodent studies following in utero exposure to TCE and its oxidative metabolites, DCA and TCA). These findings are supported by the detection of cardiac anomalies in chicken embryos exposed to TCE in ovo, and in whole embryo cultures (mouse and chicken) of TCE and/or its metabolites. In spite of the concordant evidence that TCE can be associated with cardiac defects, controversy centers on the studies by Johnson et al. (2003) and Dawson et al. (1993) and that two other well-conducted developmental toxicity studies in rats did not observe treatment-related cardiac defects following gavage or inhalation gestational exposures to TCE, i.e., in Fisher et al. (2001) and Carney et al. (2006), respectively). Detailed examination of the study protocols has identified several differences in study design and conduct, including but not limited to differences in route of administration that may have contributed to the variant study outcome. In the case of the Fisher et al. (2001) study, as previously noted, care was taken to follow the Johnson et al. (2003) fetal evaluation procedures as closely as possible, yet a number of other differences in study design and conduct remained. For example, the source of the animals, the route of exposure, the vehicle/control substance, fetal cardiac tissue preservation methods, and some fetal cardiac evaluation procedures were different. In conclusion, there has not been a confirmation of the results of the Johnson et al. (2003) and Dawson et al. (1993) studies by another laboratory, but there has also not been a repeat of the exact same study design that would corroborate or refute their findings.

Mechanistic Information and Adverse Outcome Pathway (AOP) for Cardiac Defects

A preliminary conceptual model of an AOP for TCE-induced congenital heart defects was developed, based upon data identified in a systematic literature search as well as mechanistic data that had been discussed in the 2011 IRIS assessment U.S. EPA (2011) and had provided one line of evidence regarding the potential for TCE to cause cardiac defects. Although data gaps remain, the information upon which the preliminary AOP construct is based supports the biological plausibility that TCE exposures during development can lead to disruption of key processes in the development of cardiac valves and septa.

Commonly reported cardiac defects, in humans and rodents, associated with gestational exposures to TCE and its metabolites TCA and DCA are valvuloseptal defects (atrial septal defects [ASDs], muscular and membranous ventricular septal defects [VSDs]) and pulmonary and aortic stenosis (Chiu et al., 2013; Forand et al., 2012; Yauck et al., 2004; Johnson et al., 2003; Johnson et al., 1998; Bove, 1996; Bove et al., 1995; Dawson et al., 1993, 1990; Goldberg et al., 1990; Loeber et al., 1988). The period of valvuloseptal morphogenesis defines a window of vulnerability to TCE and TCA in avian studies; thus an adverse outcome pathway (AOP) was explored relative to this dysmorphology. In normal cardiac development, valvuloseptal morphogenesis is driven by mesenchymal cells in the regions of the atrio-ventricular canal (AVC) and outflow tract (OFT) regions. AVC cushions are formed as mesenchymal cells are derived from squamosal endothelial cells (i.e., epithelial-mesenchymal transition [EMT] with an endothelial origin [EndMT]) and invade the cardiac jelly. These mesenchymal cells proliferate and differentiate into AV valves and membranous septum, as well as patterning the myocardium and acting to direct vascular flow. Evidence points to the origin of some TCE-related valvuloseptal defects through EndMT involving the following key events in the AVC and OFT cushions (Jensen et al., 2013; Kovacic et al., 2012; von Gise and Pu, 2012):

- *initiation* of EMT by signal molecules elaborated from myocardial cells into the cardiac jelly;
- *disassembly* of cell-cell junctions between squamosal endothelial cells in the endocardium;
- *delamination* by loss of polarity, cytoskeletal rearrangement, and breakdown of basal lamina;
- *invasion* of cardiac jelly by newly motile mesenchymal cells;
- *proliferation* of trans-differentiated mesenchyme to 'cellularize' and remodel the cardiac jelly;
- *patterning* of the AV myocardium by flow-mediated remodeling of the looped heart;
- *differentiation* of cardiac valves and membranous septum.

Molecular initiating events may involve a cellular initiation of vascular inflammatory signals, perhaps through an LXR/RXR-mediated effect on cholesterol homeostasis, vulnerability to reactive oxygen species (ROS) (Williams et al., 2006; Hassoun et al., 2005; Fisher et al., 2001), or disruption of the downstream consequences of VEGF signaling (Ou et al., 2003). A search of the Mouse Genome Informatics (MGI) database (http://www.informatics.jax.org/) for abnormalities in cardiac EMT identified mouse knockouts with phenotypes similar to those reported for avian studies with TCE, implicating the possibility of disruption of the following genetic signals and responses by TCE exposure during cardiac development: the TGF-beta pathway, the ephrin pathway, the Notch pathway, the VEGF pathway, and RXR signaling. In support of EndMT being a likely target, TCE exposure in chick embryos or WECs has been associated with inhibition of cell-cell separation and mesenchymal formation (Boyer et al., 2000), alterations in mesenchymal cell migration (Mishima et al., 2006; Selmin et al., 2005) and alterations in endocardial proliferation patterns (Drake et al., 2006b). In ovo studies have shown that TCE and TCA can alter cushion formation, cardiac function, and embryo survival (Drake et al., 2006a), and cushion cellularity can be altered as a function of concentration, duration, and timing of exposure, likely mediated by the ephrin-EPH system. Endocardial disruption may have additional consequences on the developing heart, related to dysregulation of cellular Ca2+ fluxes and cardiac contractility (Palbykin et al., 2011; Makwana et al., 2010; Caldwell et al., 2008; Selmin et al., 2008; Collier et al., 2003) or to alterations in cardiac hemodynamics (Rufer et al., 2010).

Weight of Evidence (WOE) Analysis

The weight of evidence for fetal cardiac defects was characterized according to the criteria described in *A Framework for Assessing Health Risk of Environmental Exposures to Children* (U.S. EPA, 2006), a scheme that is based upon principles of causality assessment developed by Hill (1965). The key components (factors) of the WOE analysis were: temporality, strength of association, variability analysis, uncertainty analysis, qualitative dose-response, experimental evidence, reproducibility (consistency), biological plausibility, alternative or multiple explanations, specificity, and coherence. Team members independently assessed the WOE, each providing evaluations based upon their expertise, and arrived at a group consensus of the evidence supporting stronger and weaker weight of association for each key factor (Table 2). In summarizing and synthesizing the WOE, the team members considered its application to decisions regarding the potential hazard for cardiac defects.

The following question framed the discussion: Does the weight of the evidence for the overall TCE database support the conclusion that TCE exposure at sufficient doses during developmental is likely to cause cardiac defects?

The majority of the team members agreed that the overall evidence in the TCE database supports a conclusion that TCE is likely to cause cardiac defects at sufficient doses when exposure occurs during a sensitive period of fetal development. This conclusion was based upon the data that demonstrate or suggest a potential hazard to cardiac development, including epidemiology studies, developmental toxicology studies in rodents with TCE and its metabolites (DCA and TCA), avian in ovo studies, in vitro assays, and mechanistic data that form the basis of a proposed AOP. It is, however, recognized that the "likely" descriptor reflects previously described limitations within the database that increase the uncertainty regarding this conclusion. The epidemiology studies provide evidence of associations between TCE, or TCE and other chlorinated solvents, and cardiac defects, but these studies have limitations related mainly to exposure measurement error and lower statistical power due to the rarity of cardiac defects. The rodent developmental toxicology studies conducted by Dawson et al. (1993), Johnson et al. (2003), and Johnson et al. (1998) that have reported cardiac defects resulting from TCE (and metabolite) drinking water exposures have study design and reporting limitations. Additionally, two good quality (GLP) inhalation and gavage rodent studies conducted in other laboratories, Carney et al. (2006) and Fisher et al. (2001), respectively, have not detected cardiac defects. These limitations and uncertainties were the basis of the single dissenting opinion of a team member regarding whether the database supports a conclusion that TCE exposures during development are likely to cause cardiac defects.

Dose–Response Analysis

Suitability of Johnson et al. (2003) study for deriving a point of departure

Given the hazard conclusion that TCE exposure during development is likely to cause cardiac defects, the next critical issue is the assessment of dose-response. The Johnson et al. (2003) study is the only available study potentially useable for dose-response analysis: it is conducted by a relevant route of exposure (drinking water), covers the appropriate developmental window (entire period of gestation), and has multiple exposure levels. A number of the study's limitations were discussed above, and will not be repeated here. Additional issues that bear additional discussion relate to the robustness of the dose-response relationship and the apparent plateau in the level of response.

There is compelling evidence of a dose-response trend in the Johnson et al. (2003) study, from a highly significant monotonic increase with dose ($P < 0.001$) based on a Cochran-Armitage trend test and from a significant trend even if the highest dose is dropped ($P < 0.04$).

The Dawson et al. (1993) study, a subset of controls plus the two highest TCE doses reported in Johnson et al. (2003), used tap water as a vehicle and drinking water source. The data from Dawson et al. (1993) were considered separately and a significant trend ($P < 0.03$) was still found.

The TCE data appear to exhibit a plateau in response around 10%. Among NTP developmental toxicology studies that exhibited a significant trend (13 of 66 studies tested for trend), none exhibited a plateau, but that could be attributable to the narrow dose spacing (2- to 4-fold) compared with the TCE study (almost 600- fold between the two highest doses, 0.218 and 129 mg/kg-day). Four of the 13 studies achieved less than 10% maximum response. For most of these dose-response curves it is impossible to determine whether response is reaching a plateau at less than 100% response. For all four having a low maximum response, there was evidence for maternal and fetal toxicity at the high doses and often at intermediate doses. (http://tools.niehs.nih.gov/ntp_tox/index.cfm?fuseaction=ntpsearch.allchemicalsforstudy&searchterm=Developmental).

On the whole, a majority of the team members agreed that the Johnson et al. (2003) is suitable for use in deriving a point of departure. The study has an appropriate design for dose-response analysis in terms of route, duration, and number dose groups. Additionally, this judgment also took into consideration the strengths and limitations of the study and uncertainties identified in the weight of evidence analysis. Additional support was derived from the finding of a robust, statistically significant dose-response relationships not only for the dataset as a whole, but also for various subsets of the dataset. Although some concern was raised regarding the plateau in the Johnson et al. (2003) response, its biological plausibility could not be ruled out based on examination of available historical developmental toxicity datasets.

Dose-Response Modeling of the Data from Johnson et al. (2003)

The 2011 TCE assessment applied the nested logistic model to the Johnson et al. (2003) data and found a $BMDL_{01}$ of 0.02 mg/kg-day. The benchmark response (BMR) of 0.01 (1%) extra risk was justified by the severity of the effect. The data were re-analyzed using this and other BMDS models to evaluate uncertainty related to model selection and modeling assumptions (http://www.epa.gov/ncea/bmds/).

Dose-response modeling of the data from Johnson et al. (2003) identified several sources of uncertainty related to modeling assumptions, presented as questions here:

(1) Does the data have a plateau at less than 100% response? The evidence is equivocal and does not permit a clear answer. A model with a plateau is plausible, but does not alter the general conclusion and results. [Note: BMDS has only one dichotomous model with a plateau that can be estimated rather than being fixed at 100%].
(2) Is it better to drop or retain the high dose? For the 2011 TCE assessment (U.S. EPA, 2011), the high dose was dropped on the strength of an examination of residuals at the low doses for the nested model. The decision to drop the high dose is confirmed in this re-examination, using non-nested dichotomous models. Dropping the high dose leads to higher model goodness of fit and better fit in the region of the BMD_{01} and BMD_{05}.
(3) Are there sufficient data in the low-dose region and near the BMD_{01} to permit reliable inference about the dose-response curve shape (which influences the BMD and BMDL)? BMD inference at the 1% extra-risk level is highly uncertain, because BMD and BMDL values vary by several orders of magnitude depending on the modeling assumptions. This is attributed to the apparent (lack of) steepness of the response curve at low doses and the lack of additional doses that would be required to fully specify curve shape in the low dose part of the curve. The data and models allow more reliable inference for BMD_{05} and BMD_{10}.

Uncertainty in the Point of Departure

There is substantial model- and parameter uncertainty at the 1% level of extra risk, although 1% is the appropriate BMR based on severity of the effect. These uncertainties can be attributed primarily to having too few data points in the low-dose range, where more data would be required to adequately characterize the dose-response shape. Uncertainty decreases for higher BMR levels (5% and 10% extra risk), although 10% exceeds the range of the data for some models. Thus, a "science-policy" choice may have to be made about a risk level and/or a POD, acknowledging the uncertainty involved. Some options for arriving at a POD include the following:

- Use the $BMDL_{01}$ (0.0207 mg/kg-day) for the nested log-logistic model selected in the 2011 TCE assessment (constrained and without the high dose group) because it provides a compromise value from the range of BMDLs derived from the variety of models examined and because EPA's science policy practices put forward in the assessment still apply.
- Use the 5% BMR for the nested log-logistic model ($BMDL_{05}$ is 0.108 mg/kg-day) and divide the resulting BMDL by an adjustment factor to extrapolate to a lower dose that is potentially protective for a smaller BMR.
- Use a model-averaged $BMDL_{01}$ or $BMDL_{05}$ for dichotomous models (using data after Rao-Scott transformation to adjust for intra-litter correlation) with the high dose dropped to achieve better fit in the low-dose range (BMD_{01} 0.0809 mg/kg-day and $BMDL_{01}$ 0.0225 mg/kg-day, BMD_{05} 0.282 mg/kg-day and $BMDL_{05}$ 0.178 mg/kg-day). (Note that this option yields results similar to that of the modeling approach used in the 2011 TCE assessment.)
- Use the LOAEL/NOAEL approach, although there is also uncertainty about defining the point of departure (POD) for this approach. Specifically, either the second highest dose (0.218 mg/kg-day) or the next lower dose (0.048 mg/kg-day) could be defended biologically as a LOAEL because the apparent extra risk values calculated from the observed responses of 2.9% and 2.5%, respectively, exceed 1%, the level identified as a suitable BMR because of the outcome.

In summary, additional dose-response analyses were performed to characterize the uncertainty in the point of departure (POD). Alternative PODs were derived based on use of alternative models, alternative benchmark response levels, or alternative procedures (such as a LOAEL/NOAEL approach), each with different strengths and limitations. These alternatives were within about an order of magnitude of the POD derived in the 2011 TCE assessment.

Given the numerous uncertainties in the dose-response analysis derived from both the nature of the data and from constraints inherent in BMD modeling programs and procedures, the majority of team members considered the confidence in the point of departure based on (Johnson et al., 2003) to be between "low" and "medium." Overall, however, the team members concluded that the point of departure derived in the 2011 TCE assessment, which used an approach consistent with standard U.S. EPA dose-response practices, remained a reasonable choice.

Summary/Conclusions

This updated analysis of the potential for exposure to TCE during critical windows of development to result in cardiac defects was conducted as an independent review by expert multidisciplinary EPA scientists from ORD/NCEA, ORD/NHEERL, and NCCT. This review resulted in: 1) an updated characterization of uncertainties in the TCE database for cardiac defects, 2) documentation of data and weight of evidence evaluation, 3) an extended characterization of uncertainties in the dose-response modeling, and 4) an expanded consideration of the mechanistic database that may support future research to develop an adverse outcome pathway for cardiac defects resulting from TCE exposures. Key conclusions of the team include:

- Six epidemiological studies are available on total or specific cardiac defects and TCE exposure; five of the six studies were reviewed in the EPA 2011 Trichloroethylene Toxicological Review (U.S. EPA, 2011). One study did not report on total cardiac defects and was not included in the review. All studies have limited sensitivity for detecting an association between exposure to TCE and other chlorinated solvents and cardiac defects in offspring. One study did not observe an association, likely a result of lower sensitivity and statistical power. Two studies provide evidence for an association between maternal exposure to TCE or TCE and other chlorinated solvents in drinking water and cardiac defects. Two other studies with greater potential for biases observed elevated risk estimates between TCE exposure and cardiac defects in offspring, and provide supportive evidence.
- Alterations in fetal cardiac development have been observed in two rodent studies following in utero exposure to TCE and in four studies (of three laboratories) following exposure to its oxidative metabolites, DCA and TCA). These findings are supported by the detection of cardiac anomalies in three studies (conducted in two laboratories) that exposed chick embryos to TCE in ovo, and in two whole embryo culture studies (mouse and chicken), conducted in two laboratories, of TCE and/or its metabolites.
- An evaluation of the Johnson et al. (2003) and Dawson et al. (1993) study (and published errata) identified and characterized both strengths and limitations in the study design and reporting. Strengths of this study include the examination of fetal hearts without knowledge of treatment (or control) group, standardized methods of fetal evaluation, examination of the gross (in situ) and internal structure of the fetal hearts by a group of 3 senior researchers, confirmation of cardiac anomalies by consensus agreement, and that the researchers shared individual fetal and litter cardiac abnormality data for treated groups with EPA, thereby facilitating independent statistical analysis of the data. Limitations include reporting deficits and study design issues (e.g., the study was conducted over a period of 6 years, combined control data were used for comparison to treated groups, and concerns about possible imprecision in exposure characterization)
- Two other high quality rodent developmental toxicity studies did not identify cardiac fetal defects following exposure to TCE by gavage or inhalation ((Fisher et al., 2001) and (Carney et al., 2006), respectively). It is not clear why there is a difference in outcomes between the Johnson et al. (2003) study versus the Fisher and Carney studies, which did not detect cardiac defects. There were differences in study design and conduct, in spite of efforts by Fisher et al. (2001) to minimize such differences, but the team cannot determine from available data the reason that outcomes differed across these studies.

- A preliminary conceptual model of an Adverse Outcome Pathway (AOP) for TCE-induced congenital heart defects supports the biological plausibility that TCE exposures during development can lead to disruption of key processes in the development of cardiac valves and septa. The stage of development for such defects is conserved across species, hence supporting relevance of the chicken embryo in ovo and mouse and chicken whole embryo findings.
- A weight of evidence evaluation of the TCE database based on the criteria described in *A Framework for Assessing Health Risk of Environmental Exposures to Children* (U.S. EPA, 2006) supports a conclusion that TCE is likely to cause cardiac defects at sufficient doses when exposure occurs during a sensitive period of fetal development.
- The weight of evidence for developmental cardiac toxicity based on the *Guidelines for Developmental Toxicity Risk Assessment* (U.S. EPA, 1991) categorized as "Sufficient Experimental Animal Evidence" and "Limited Human Data."
- The Johnson et al. (2003) study data show a statistically-significant trend with dose. An expanded dose-response analysis of the Johnson et al. (2003) data using additional models illuminates some of the model and parameter uncertainty in the derivation of a BMDL to use as a point of departure; however, the range of results supports the findings of the 2011 TCE assessment, which used an approach consistent with EPA science practices.
- A number of different approaches could yield points of departure (PODs) for a 1% extra risk within about an order of magnitude of the BMDL value used in the 2011 IRIS assessment. (The BMDL in the 2011 IRIS assessment was 0.021 mg/kg-day using a target BMR of 1%.)
- Based on these conclusions, the hazard and dose-response database for developmental cardiac toxicity is categorized as "Sufficient Experimental Animal Evidence" and "Limited Human Data" in accordance with the *Guidelines for Developmental Toxicity Risk Assessment* (U.S. EPA, 1991).

The database was considered by the majority of team members to be adequate in support of the categorization of the health-related database for hazard and dose-response (U.S. EPA, 1991), with the determination that there was "Sufficient Experimental Animal Evidence" and "Limited Human Data" for developmental cardiac toxicity. According to the Guidelines, this category "includes data from experimental animal studies and/or limited human data that provide convincing evidence for the scientific community to judge that a potential for developmental toxicity exists." The minimum evidence that would be necessary to determine whether there is or is not sufficient evidence of developmental toxicity is the existence of appropriate, well-conducted animal study(ies). The overall TCE database met this criterion, although limitations and uncertainties in the primary study used in dose response (Johnson et al., 2003) are acknowledged. Those limitations and uncertainties were the basis of the only dissenting opinion (i.e., of one team member) regarding whether the database supports a conclusion that TCE exposures during development are likely to cause cardiac defects.

The team had a range of views as to their confidence in the conclusion regarding hazard for cardiac defects – with three out of nine scientists expressing an opinion concluding the confidence should be medium to high, and six of nine concluding confidence should be "low" or "medium." These ratings were influenced by whether the primary focus was on the uncertainties and limitations of the Johnson et al. (2003) study or whether it was on the weight of evidence consideration of the entire database. The 2011 IRIS TCE assessment did not assess the confidence in the hazard for cardiac defects independently, but judged the confidence in overall developmental toxicity to be "medium-to-high". It also stated that the overall weight of evidence supported an effect of TCE on cardiac development, although recognizing that the Johnson et al. (2003) study has "important limitations".

Overall, taking into account the study's design, its strengths and limitations, and uncertainties in the weight of evidence, a majority of the team members agreed that the Johnson et al. (2003) study was suitable for use in deriving a point of departure. However, confidence of team members in the dose response evaluation of the cardiac defect data from the Johnson et al. (2003) study was characterized as between "low" and "medium" (with 7 of 11 team members rating confidence as "low" and four team members rating confidence as "low to medium"). Nonetheless, the team members concluded that the point of departure derived in the 2011 TCE assessment, which used an approach consistent with standard U.S. EPA dose response practices, remained a reasonable choice. The IRIS TCE assessment (U.S. EPA, 2011) indicated "moderate" confidence in the candidate reference values for developmental cardiac effects (pp. 5-96 and 5-100) and "high" confidence for the non-cancer reference values based upon multiple effects which included the developmental cardiac defects (p. 6-43). The majority of the team agreed that the results of the present analysis are consistent with the dose-response conclusions of the 2011 IRIS TCE assessment.

Future Research: Future research should address the recommendations of the NRC (2006). Three general areas might be explored: 1) In vivo studies should be conducted to evaluate the lowest-observed-adverse-effect levels and mode of action for TCE-induced developmental function and morphology, gain information on metabolic activation in the avian model for the evaluation of interspecies differences and determine the most appropriate species for human modeling, evaluate tissue-specific concentrations of TCE and its metabolites. Advanced imaging techniques (e.g., ultrasound, micro-CT) could be utilized. 2) Research is needed to explore linkages between proposed adverse-outcome-pathways and TCE, TCA, and DCA exposure in mammals. Research should be conducted to characterize susceptible populations, including the influence of genetic polymorphisms or maternal age as risk factors for TCE-induced adverse developmental effects. 3) Additional research is needed to better characterize human exposures and outcomes. An epidemiology research program should include the examination of TCE and other solvents in large case-control studies of congenital malformations that include cardiac defects, such as the Center for Disease Control's National Birth Defects Prevention Study (http://nbdps.org/).

Team Members:

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- Barbara Abbott, Sid Hunter, Michael Narotsky (NHEERL, ORD)
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Table 1. WOE Evaluation of the Potential for Development Exposures to TCE to Result in Cardiac Defects					
Key Factor [a,b]	**Type of evidence to consider**	**Data**	**Evidence for stronger weight of association**	**Evidence for weaker weight of association**	**Comments or Null Evidence**
Temporality	Timing of exposures and response	Tox	Studies in various species in which TCE (or metabolites DCA or TCA) were administered during a sensitive period of in utero cardiac development resulted in morphological and/or functional alterations. • Drinking water administration of TCE to rats on GD 1-22 resulted in a statistically significant treatment-related increase in the incidence of cardiac defects (Johnson et al., 2003; Dawson et al., 1993). • Drinking water administration of TCA (the TCE oxidative metabolite) to rats on GD 1-22 resulted in a statistically significant treatment-related increase in the incidence of cardiac defects (Johnson et al., 1998). Gavage administration of TCE metabolites (DCA and TCA) on GD 6-15 (Smith et	Some in vivo or in vitro studies rodent studies in which TCE (or metabolites DCA or TCA) was administered during a sensitive period of in utero cardiac development resulted in no morphological alterations. • Gavage administration of TCE or metabolites (DCA and TCA) to rats on GD 6-15 did not result in treatment-related cardiac defects (Fisher et al., 2001). • Inhalation exposures of TCE to rats on GD 6-20 (Carney et al., 2006) or to rats and mice on GD 6-15 (Schwetz et al., 1975) did not result in treatment-related cardiac defects.	

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			al., 1992, 1989) or of DCA during discrete windows of time within GD 6-15 (Epstein et al., 1992) resulted in treatment-related increases in the incidences of cardiac defects. • Avian in ovo studies that administered TCE or TCA during the period of valvuloseptal morphogenesis (e.g., HH 15-20) resulted in altered cardiac morphology and/or function (Rufer et al., 2010; Drake et al., 2006a; Loeber et al., 1988). • A study of DCA exposure to zebra fish (Hassoun et al., 2005) demonstrated evidence of a disruption in cardiac development (pericardial edema and altered heart rate). • Mouse whole embryo culture studies of DCA and TCA administered at the period of 3-6 somites detected cardiac defects (Hunter et al., 1996); a chicken whole embryo culture study of TCE administered at HH 13-14		

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			detected alterations in AV cushion (Mishima et al., 2006). • Avian atrioventricular canal cell culture (HH 16) study found evidence of inhibited endothelial cell separation and early events of mesenchymal cell formation in the heart following TCE exposures (Boyer et al., 2000).		
	Exposure occurs before outcomes onset	Epi	• Four cohort or case-control studies consider temporality (Ruckart et al., 2013; Forand et al., 2012; Yauck et al., 2004; Goldberg et al., 1990). Three studies observe an association between the TCE exposure surrogate and major cardiac defects (Forand et al., 2012; Yauck et al., 2004; Goldberg et al., 1990). An association with conotruncal defects, specifically, observed in Forand et al. (2012).	• Temporality was not considered in Bove (1996)/Bove et al. (1995), Goldberg et al. (1990), or Lagakos et al. (1986).	• The small numbers of conotruncal heart defects in Ruckart et al. (2013) precluded any analysis of this endpoint and TCE exposure.
Strength of association	Study quality, including study strengths and	Tox	• For Johnson et al. (2003), Dawson et al. (1993), and Johnson et al. (1998), all of	• For Johnson et al. (2003) major study quality limitations include the use of data pooled	• Some studies that reported no cardiac defects following

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	limitations		which detected cardiac malformations, study quality strengths include randomized assignment to test group, detailed description of fetal cardiac dissection and evaluation procedures, evaluation of fetal hearts without knowledge of treatment group, and confirmation of all cardiac defects by consensus of 3 experts. Statistical analysis of data from this study was appropriately conducted by EPA statisticians using individual fetal and litter data that were provided by the study author. • The power of detection in the Johnson et al. (2003) study was enhanced by the use of historical controls that did not demonstrate a temporal shift in cardiac defects. A significant dose related trend in cardiac defects was observed even without large group sizes. • A strong association of	from separate study cohorts conducted over an approximately 6-year period, the use of tap water as the vehicle for some of control and treated groups (as reported by Dawson et al. (1993) with no characterization of possible contaminants and incomplete reporting of study methods and results. • While Dawson et al. (1993) indicated that levels of TCE in dose formulations were tested by gas chromatography, the analytical findings were not reported. Johnson et al. (2003) did not report whether dose formulations were analyzed. Further, levels of TCE were not assessed in the vehicle control water; therefore, it is plausible that TCE contaminated the water and that doses were actually higher than measured. • The Dawson et al. (1993) and Johnson et al. (2003) studies estimated doses based on the average water consumption. This method does not provide	TCE gestational exposures (Narotsky and Kavlock, 1995; Narotsky et al., 1995; Healy et al., 1982; Hardin et al., 1981) or avian in ovo studies (Bross et al., 1983; Elovaara et al., 1979) did not indicate that detailed evaluation of fetal hearts was conducted. • A rat whole embryo culture study of TCE administered at the period of 4-7 somites detected no cardiac defects in a study by (Saillenfait et al., 1995); however, the study methods indicate that there was no evaluation of the embryonic heart.

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			exposure to response was observed at high dose levels in multiple studies that identified cardiac defects. In Johnson et al. (2003) there was a highly significant positive trend for cardiac defects. • Potential confounding factors exist in studies that did not identify cardiac defects (e.g., different routes of exposure, the use of different rodent strains or suppliers across studies, and the use of soybean oil as a vehicle in Fisher et al. (2001).	precise information to calculate TCE dose because variability in drinking water consumption among dams is not characterized. • The dose selection for Johnson et al. (2003) resulted in a NOAEL that is approximately 700-fold lower than the next highest dose. • Some studies that did not identify treatment-related cardiac defects following developmental exposures to TCE, e.g., Carney et al. (2006), Fisher et al. (2001), and Schwetz et al. (1975), were well-conducted and adequately-reported GLP and/or guideline studies with no substantive limitations identified. • One study Fisher et al. (2001) attempted to replicate the methods used in the Johnson et al. (2003) study, utilizing the same fetal cardiac dissection and evaluation techniques, and including one of the Johnson et al. (2003) study authors in the assessment team, yet found no	

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				treatment-related cardiac defects.	
	Magnitude of the effect measure	Epi	• Increased risk estimates between all or major cardiac defects ranged from 1.24 (95% CI: 0.75, 1.94) to 2.40 (95% CI: 1.27, 3.62) observed in 3 studies (Forand et al., 2012; Bove, 1996; Bove et al., 1995; Goldberg et al., 1990). Stronger associations, observed with the TCE exposure surrogate for conotruncal defects and ventricular septal defects than for major cardiac defects, a broader category (Forand et al., 2012; Bove, 1996; Bove et al., 1995). A fourth study observed an increased risk estimate of 6.2 (95% CI: 2.6, 14.5) for cardiac defects in infants of mothers aged ≥38 years and maternal residence within 1.32 miles from at least one TCE emissions source (Yauck et al., 2004).	• No association in Yauck et al. (2004) in mothers <38 years of age and maternal residence within 1.32 miles from at least one TCE emissions source nor in Lagakos et al. (1986), which does not observe an association with cardiac defects. Alternative reasons such as lower statistical power may explain these observations.	•
Variability analysis	Sources of within- and	Tox	• Johnson et al. (2003) test subject source, husbandry, and randomization	• The Johnson et al. (2003) study reported data from several cohorts of animals, which were	• Based upon the toxicokinetic profile of TCE (U.S. EPA,

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	cross-study variability that contribute to uncertainty		procedures were consistent across all cohorts, i.e., including Dawson et al. (1993) and metabolite studies Johnson et al. (2003). Fetal cardiac evaluation methodology, which included evaluation without knowledge of treatment group and confirmation of all cardiac anomalies by 3 expert scientists, was also consistently applied across cohorts and studies from the UAZ laboratory. This had the result of reducing intra- and inter-study variability in the assessment. • Johnson et al. (2003) reported that cardiac defect incidences were consistent across all control cohorts (55 litters over approximately 6 years). An EPA review of the available control data did not observe unusual heterogeneity in prevalence of malformations. • Studies that reported cardiac defects following	on study over a period of approximately 6 years. The data included control cohorts, some of which were concurrent and some that were non-concurrent to the TCE-treated groups (Johnson et al., 2014, 2005). Data that definitively link the individual control litter response data with each particular cohort are no longer available for independent examination. • Different study outcomes were observed in studies that had many similarities in study design and conduct, i.e., Dawson et al. (1993) and Johnson et al. (2003) identified exposure related cardiac defects while Fisher et al. (2001) did not. In the Fisher et al. (2001) study, care was taken to ensure that the same cardiac evaluation methods were used as in the Dawson et al. (1993) and Johnson et al. (2003) studies, including fetal evaluation with knowledge of treatment group, and one of the study authors of	2011), it is considered unlikely that toxicokinetic factors contributed significantly to differences in response across study protocols.

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			administration of metabolites (DCA and TCA) used randomized assignment of maternal animals to test group, thus reducing intra-study variability. • Although Dawson et al. (1993) and Johnson et al. (2003) identified cardiac defects following exposures to TCE during development, Fisher et al. (2001), Carney et al. (2006), and Schwetz et al. (1975) did not find treatment-related cardiac abnormalities. This may be the result of differences in the study design and assessment methods. This includes such aspects as animal strain, age, source, exposure route and vehicle, duration of exposure, and cardiac evaluation methods.	Johnson et al. (2003) participated in the fetal examination. • The use of soy bean oil in the Fisher et al. (2001) study vs. water vehicle and control for Johnson et al. (2003) and Dawson et al. (1993) studies. • The Johnson et al. (2003) and Dawson et al. (1993) studies did not calculate variability in TCE dose by measuring individual dam water consumption.	
	Sources of within- and cross-study variability that contribute to	Epi	• NE (not considered in Hill analysis)	• NE (not considered in Hill analysis)	• Studies examined different populations, exposure levels, gradients, and media. Additionally, different sets of

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	uncertainty				strengths and uncertainties in this set of studies would contribute to observed cross-study variability.
Uncertainty analysis	Missing information or data gaps, within and across studies	Tox	• For the studies conducted by the UAZ laboratory (Johnson et al., 2003; Johnson et al., 1998; Dawson et al., 1993) that identified cardiac defects following exposures to TCE, DCA, or TCA, detailed descriptions of evaluation methods for assessment of cardiovascular effects were provided. • Individual fetal and litter cardiac findings data, as well as detailed information on study conduct and fetal evaluation methods, were provided to the EPA for (Dawson et al., 1993) and (Johnson et al., 2003).	• The publications for studies conducted by the UAZ laboratory that identified cardiac defects following exposures to TCE, DCA, or TCA (Johnson et al., 2003; Johnson et al., 1998; Dawson et al., 1993) did not report essential study details, and generally did not include summaries of maternal data or fetal data for endpoints other than cardiac defects. • For well-conducted studies that did not detect cardiac defects following developmental exposures to TCE or metabolites (Carney et al., 2006; Fisher et al., 2001) adequate descriptions of study methodology and summary data for maternal and fetal findings were reported. • Mechanistic data for alterations	•

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				in cardiac development are limited and do not identify initiating events for the putative AOP.	
	Missing information or data gaps, within and across studies	Epi	• NE (not considered in Hill analysis)	• NE (not considered in Hill analysis)	•
Qualitative dose-response	Association between exposure/dose and degree of effect	Tox	• Alterations in cardiac development were observed in multiple studies at high dose levels following TCE, DCA, or TCA exposures (Johnson et al., 2003; Johnson et al., 1998; Dawson et al., 1993; Smith et al., 1992, 1989). • The incidence of cardiovascular effects increased as a function of dose in Johnson et al. (2003). • An association between exposure to TCE (or DCA or TCA) and alterations in cardiac development was reported in various animal models, i.e., LE and SD rats,	• The dose response for cardiac defects identified by Johnson et al. (2003) could only be fit to a model with elimination of the high dose data from the analysis. The lowest dose tested had a zero response for cardiac defects, below the historical control incidence. The doses tested were spaced over several orders of magnitude, with wide gaps. • Carney et al. (2006) was the only other study in the database that evaluated developmental effects of TCE over multiple dose levels. In that study, no fetal toxicity and minimal maternal toxicity was reported.	• TCE doses tested in Dawson et al. (1993) and Johnson et al. (2003) (drinking water): 2.5 ppb, 250 ppb, 1.5 ppm, or 1100 ppm (0, 0.00045, 0.048, 0.218, or 129 mg/kg-day) • TCE doses tested in Fisher et al. (2001) (gavage): 500 mg/kg-day • TCE doses tested in Carney et al. (2006) (inhalation): 50, 150, or 600 ppm (268.5, 805.5, or 3222 mg/m^3)

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			CD-1 mice, chicken embryos, and zebra fish (Drake et al., 2006b; Drake et al., 2006a; Williams et al., 2006; Hassoun et al., 2005; Johnson et al., 2003; Dawson et al., 1993; Smith et al., 1992, 1989). • A BMDL for Johnson et al. (2003) was derived by EPA statisticians from individual cardiac defect data provided to EPA. Litter contribution to the outcome of interest was incorporated in the analysis. A significant dose-response trend was identified, whether or not the high dose value was included in the analysis.		
	Exposure-response gradient: Association between exposure/dose and degree of effect	Epi	• NE	• Goldberg et al. (1990) and Lagakos et al. (1986) examined exposure-response; none observed.	•
Experimental	Hypothesis Tox		• A study by (Epstein et al.,	• Studies in rodents that	• Studies that

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evidence	testing: manipulation of exposure scenario with resulting alterations in response		1992) administered the metabolite DCA to rats on varied days of gestation and identified critical windows of exposure for eliciting cardiac developmental defects. • No statistically significant increases in congenital heart defects were observed in groups of rats that were exposed to TCE prior to pregnancy only (Dawson et al., 1993). • (Drake et al., 2006b) demonstrated that cardiac defects did not occur in chick embryos exposed to TCE and TCA during the period of cardiac specification (approximately GD 6 in rats) rather than the period of valvuloseptal morphogenesis.	administered TCE via drinking water detected an increase in fetuses with cardiac defects (Johnson et al., 2003; Dawson et al., 1993); studies that administered TCE via other routes (gavage and inhalation) were negative for this response(Carney et al., 2006; Fisher et al., 2001; Schwetz et al., 1975). • In a whole embryo culture (WEC) study of DCA and TCA (Hunter et al., 1996), that identified cardiac defects, the acid nature of DCA and TCA may have impacted dysmorphogenesis.	manipulated the gestational exposure period were not conducted with TCE.
	Association not observed once exposure ceases	Epi	• NE	• No differences between observed and expected numbers of cardiac defect cases once wells were closed in contaminated area (Goldberg et al., 1990).	•
Reproducibility	Reproducibility:	Tox	• Studies that administered	• Studies conducted in other	• Studies that did not

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[Consistency]	Corroboration across studies, labs, routes of exposure, species, etc.		TCE in drinking water to rats on GD 1-22 were conducted over a period of approximately 6 years by researchers at the same academic facility (UAZ, Tucson) used the same cardiac evaluation methods and identified treatment and dose-related cardiac malformations (Johnson et al., 2003; Johnson et al., 1998; Dawson et al., 1993). A preliminary screening study that utilized intrauterine administration of TCE also detected cardiac defects (Dawson et al., 1990). The types of cardiac malformations observed were similar across study cohorts and treatment groups throughout the duration of the research program. • Studies on TCE metabolites (TCA and TCA) conducted in other laboratories (Epstein et al., 1992; Smith et al., 1992, 1989) identified cardiac defects similar to those	laboratories than UAZ and that administered TCE by gavage or inhalation (Carney et al., 2006; Fisher et al., 2001; Schwetz et al., 1975) did not identify statistically significant increases in cardiac defects. Fisher et al. (2001) used the same cardiac evaluation methods as the UAZ lab.	identify cardiac defects with TCE and/or metabolite exposures (Carney et al., 2006; Fisher et al., 2001; Schwetz et al., 1975) did not replicate all aspects of the Johnson et al. (2003) study, even though Fisher et al. (2001) used the same cardiac evaluation techniques as (Johnson et al., 2003) and Dawson et al. (1993), and therefore provide only limited evidence of lack of reproducibility.

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			observed in the UAZ studies. • Cardiac septal anomalies were observed in avian in ovo studies (Rufer et al., 2010; Drake et al., 2006a), and in WEC assays (Mishima et al., 2006; Hunter et al., 1996) with TCE and/or metabolite exposures. Zebrafish studies also demonstrated evidence of alterations in cardiac development (Williams et al., 2006; Hassoun et al., 2005).		
	Consistency: Association observed in different populations, places, time and circumstances.	Epi	• Association between cardiac defects and TCE exposure surrogate observed in four studies. These studies were of different populations living in different state (NY, NJ) and covered slightly different time period (1983-2000, 1985-1988) (Forand et al., 2012; Bove, 1996; Bove et al., 1995). Two other studies of weaker designs were of different populations and carried out in two different locations in the United States, and provide supporting evidence (Yauck	• Lagakos et al. (1986) compared a pregnancy receiving contaminated residential well water to a pregnancy not receiving residential water from contaminated wells and does not observed an association between cardiac defects and contaminated drinking water.	•

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			et al., 2004; Goldberg et al., 1990).		
Biological plausibility	Observed outcome can be attributed to toxic insult given the known science	Tox	• Avian in ovo studies and atrioventricular cell culture studies support the biological plausibility of effects of TCE on cardiac development, given that early chick heart development is similar to mammalian (including human), particularly regarding the role of the cardiac cushion in septation (NRC, 2006). • Preliminary exploration of a possible adverse outcome pathway (AOP) has resulted in a reasonable conceptual model for TCE-induced congenital heart defects. In this construct, the vulnerable period is defined by endocardial morphogenesis. Endothelial–mesenchyme transition is disrupted in the area of the atrioventricular canal, leading to septal defects. Possible genetic contributions to abnormal cardiac development include	• A definitive AOP for TCE-induced cardiac defects, including a putative initiating event, has not yet been characterized. Additional mechanistic data are needed to support the hypothesized AOP. • There are insufficient mechanistic data to characterize additional potential MOAs other than that hypothesized in the AOP.	• It is possible that multiple modes of action are involved in alterations to cardiac development.

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Key Factor [a,b]	**Type of evidence to consider**	**Data**	**Evidence for stronger weight of association**	**Evidence for weaker weight of association**	**Comments or Null Evidence**
			disruption of TGF-beta pathway, endrin pathway, Notch pathway, VEGF pathway, and RXR signaling. At a cellular level, epithelial-mesenchymal transition may be affected in the endocardium, at the tissue level, there is altered cellularity of the endocardial cushion, and secondary effects such as dysregulation of cellular Ca^{2+} fluxes may result in additional impacts on the developing heart.		
	Observed association plausible given the known science	Epi	• NE	• NE	• In vitro and in vivo animal studies report cardiac defects with TCE and TCE-metabolite exposure.
Alternative or multiple explanations	Other possible explanations for observed outcome after the exposure of interest	Tox	• Given the presumed contribution of both environmental exposures and genetic predisposition in human congenital heart disease (Richards and Garg, 2010), it is possible that the test subjects used in the	• There is a possibility that cardiac defects detected in the Dawson et al. (1993) study were associated in part with the use of tap water as a control vehicle (i.e., possible presence of contaminants).	•

Table 1. WOE Evaluation of the Potential for Development Exposures to TCE to Result in Cardiac Defects					
Key Factor [a,b]	**Type of evidence to consider**	**Data**	**Evidence for stronger weight of association**	**Evidence for weaker weight of association**	**Comments or Null Evidence**
			Johnson et al. (2003) study and others conducted in that laboratory may have been particularly susceptible to alterations in cardiac development. • Other contributing factors or confounding factors were not specifically identified in the evaluated in-vivo studies. • It is possible that the absence of treatment-related cardiac defects in well-conducted TCE studies (Carney et al., 2006; Fisher et al., 2001) or metabolite studies (Fisher et al., 2001) was due to confounding variables such as differences in strain/source of animal model, route of exposure, toxicokinetics, vehicle [e.g., soybean oil in Fisher et al. (2001)], or differences in cardiac evaluation methods. • It is unlikely that the cardiac defects observed by Johnson et al. (2003) were an artifact of the evaluation procedures used, since a study by Fisher		

Table 1. WOE Evaluation of the Potential for Development Exposures to TCE to Result in Cardiac Defects					
Key Factor [a,b]	**Type of evidence to consider**	**Data**	**Evidence for stronger weight of association**	**Evidence for weaker weight of association**	**Comments or Null Evidence**
			et al. (2001), using the same fetal cardiac evaluation procedures, did not identify an association between TCE exposure and the incidence of cardiac defects.		
	Other possible explanations for observed outcome after the exposure of interest (not considered in Hill analysis)	Epi	• Potential maternal risk factors were adjusted in statistical analysis in Forand et al. (2012) and Yauck et al. (2004) or were not found in statistical analyses to influence observed association by +15% (Bove, 1996; Bove et al., 1995).	• Potential for confounding from another exposure given the poor exposure definition in Yauck et al. (2004). The positive association in Goldberg et al. (1990) may result from likely selection biases in controls.	•
Specificity	Single cause and effect relationship resulting from exposure to test substance	Tox	• Cardiac defects in rats appear to be attributable to direct chemical exposure to TCE or metabolites (DCA or TCA) and are unlikely to be the result of secondary effect of maternal toxicity. Johnson et al. (2003) reported that TCE exposure via drinking water to pregnant rats did not result in maternal toxicity. Carney et al. (2006) reported minimal decreases in body weight gain in dams, with no adverse fetal	• Studies conducted in other laboratories than UAZ and that administered TCE by gavage or inhalation (Carney et al., 2006; Fisher et al., 2001; Schwetz et al., 1975) did not identify cardiac defects. Fisher et al. (2001) used the same cardiac evaluation methods as the UAZ lab. • The cardiac defects detected in the Dawson et al. (1993) study may have been related to the use of tap water as a vehicle	•

Table 1. WOE Evaluation of the Potential for Development Exposures to TCE to Result in Cardiac Defects					
Key Factor [a,b]	**Type of evidence to consider**	**Data**	**Evidence for stronger weight of association**	**Evidence for weaker weight of association**	**Comments or Null Evidence**
			outcomes. In fetuses, there was no indication of TCE-related fetal weight deficits, external or skeletal anomalies, or of soft tissue alterations other than cardiac defects in Johnson et al. (2003), nor in any other study. • The majority of the cardiac malformations following TCE exposures to rats (Johnson et al., 2003; Dawson et al., 1993) or chicks (Rufer et al., 2010; Drake et al., 2006a) during sensitive periods of cardiac development were ventricular septal defects, valve defects, or outflow tract abnormalities. Mechanistic data suggest a common etiology (disruption of the cardiac cushion formation) for the observed cardiac defects Boyer et al. (2000).	(i.e., possible contaminants).	
	Single cause and effect relationship	Epi	• NE	• Specificity not a critical compared to other Hill aspects since outcomes may have several risk factors. Maternal	•

Table 1. WOE Evaluation of the Potential for Development Exposures to TCE to Result in Cardiac Defects					
Key Factor [a,b]	**Type of evidence to consider**	**Data**	**Evidence for stronger weight of association**	**Evidence for weaker weight of association**	**Comments or Null Evidence**
	resulting from exposure to test substance			risk factors, specifically chemical risk factors, associated with cardiac defects in infants have not been well studied.	
Coherence	Summary: Extent to which data are similar in outcome and exposure across database	Tox	• Multiple studies were conducted at UAZ (Johnson et al., 2003; Johnson et al., 1998; Dawson et al., 1993), in which rats were administered TCE or metabolites DCA or TCA in drinking water on GD 1-22 and for which study design and cardiac evaluation methodologies were consistent. The outcomes of these studies (detection of cardiac defects, particularly septal defects, valve abnormalities, and outflow tract anomalies) are consistent across these studies. Additionally, these outcomes are supported by the results of avian in ovo and in vitro studies, studies with TCE metabolites (DCA and TCA) in rodents, in vitro whole embryo culture studies,	• Developmental toxicity studies with TCE that were conducted in other laboratories (Carney et al., 2006; Fisher et al., 2001; Schwetz et al., 1975) administered TCE to rats of other strains or sources, using different routes of exposure (inhalation or gavage), administered on different days of gestation (i.e., not including GD 1-6) than the UAZ studies and did not identify cardiac defects. No other study in the TCE database reported cardiac defects at the low dose levels reported by Johnson et al. (2003).	•

Table 1. WOE Evaluation of the Potential for Development Exposures to TCE to Result in Cardiac Defects					
Key Factor [a,b]	**Type of evidence to consider**	**Data**	**Evidence for stronger weight of association**	**Evidence for weaker weight of association**	**Comments or Null Evidence**
			and mechanistic data.		
	Cause and effect interpretation should not conflict with the generally known facts of the natural history and biology of the disease	Epi	• Associations in epidemiologic studies of cardiac defects and maternal occupational exposure to degreasing solvents or to organic solvents (Gilboa et al., 2012; Loffredo et al., 1991; Tikkanen and Heinonen, 1991; Tikkanen and Heinonen, 1988).	• NE	•

NE = No relevant evidence.

HH = Hamburger-Hamilton stages of chick development (Hamburger and Hamilton, 1951).

Tox = Animal toxicology studies; Epi = Epidemiological studies

Key Factor references:

a U.S. EPA (2006)

b Hill (1965)

Table 2. Comparison of TCE Prenatal Developmental Toxicity Study Methods				
	Dawson et al. (1993)	Johnson et al. (2003)	Fisher et al. (2001)	Carney et al. (2006)
Study Description/Objective				
GLP; guideline [G], guideline-type [GT], or research [R] protocol	R	R	R	GLP, G
Test Subjects				
Species	Rat	Rat	Rat	Rat
Strain	SD	SD	SD	SD
Source (company)	Harlan	Harlan	CRL	CRL
Source (location	Indianapolis, IN	Indianapolis, IN	Raleigh, NC	Portage, MI
Dates of study conduct	1989-1990	1989-1995	NR	NR
Day of mating confirmation (GD 0 or GD 1)	NR	NR	GD 0	GD 0
Day of cesarean section	GD 22	GD 22	GD 21	GD 21
Treatment				
Test Substance	TCE	TCE	TCE	TCE
Source	Aldrich	Aldrich	Aldrich	Dow
Purity (%)	NR	NR	NR	99%
Negative control (vehicle)	Tap water	Distilled water	Soybean oil	Ambient air
Positive control	N	N	RA	N
No. of treated groups	2	4	1	3
Group size (litters/group)	9-15	9-12	19-25	27
Random assignment of test subjects to groups	Y	Y	Y	Y

Table 2. Comparison of TCE Prenatal Developmental Toxicity Study Methods				
	Dawson et al. (1993)	Johnson et al. (2003)	Fisher et al. (2001)	Carney et al. (2006)
Dose period (duration, gestation-only groups)	GD 1-22	GD 1-22	GD 6-15	GD 6-20
Daily dosing schedule	Ad libitum	Ad libitum	1x/day	6 hrs/day, 7 days/wk
Route of administration	DW	DW	Gavage	Inhalation a
Maternal evaluation				
In-life data (BW, FC, WC, and/or clinobs)	Y	Y	Y	Y
Postmortem data (necropsy, organ wts, pathology, and/or CL)	Y	Y	Y	Y
Fetal evaluation				
Implantations and resorptions (early and late)	Y	Y	Y	Y
Fetal weight, length, sex	Y	Y	Y	Y
External fetal exam	Y	Y	Y	Y
Percent fetuses (litters) evaluated for external findings	100 (100)	100 (100)	100 (100)	100 (100)
Visceral examination	Y	Y	Y	Y
Percent fetuses (litters) evaluated for visceral findings	100 (100)	100 (100)	100 (100)	50 (100)
Fresh dissection (in situ organ examination)	Y (heart)	Y (heart)	Y (heart)	Y (viscera)
Wilson exam (Bouins fixation, free-hand sections)	N	N	N	Y (head)

Table 2. Comparison of TCE Prenatal Developmental Toxicity Study Methods				
	Dawson et al. (1993)	Johnson et al. (2003)	Fisher et al. (2001)	Carney et al. (2006)
Fetal cardiac examination methods	Y	Y	Y	Y
Fresh dissection and evaluation	UAZ method	UAZ method	UAZ method	Staples exam
Free-hand section of decalcified fetuses	N	N	N	N
Preservation	GLA flush & immersion	GLA flush & immersion	formalin immersio n	NR
Confirmation of findings Y b		Y b	NR	NR
Skeletal examination	NR	NR	NR	Y
Percent fetuses (litters) evaluated for skeletal findings	NR	NR	NR	50 (100)
Bone development	NR	NR	NR	Y
Cartilage development	NR	NR	NR	Y
Random selection of fetuses for visceral or skeletal evaluation	NA	NA	NA	NA
Assessment of fetuses without knowledge of treatment group	Y	Y	Y	Y
Footnotes:				
This table only includes mammalian studies with prenatal TCE exposures and an evaluation of fetal morphology.				
NR = not reported; NA = not applicable; Y = yes, N = No; DW = drinking water; GD = gestation day; RA = retanoic acid; GLA = gluteraldehyde				
Test subject strain: SD = Sprague-Dawley				
Test subject source: CRL = Charles River Laboratories; Harlan = Harlan Laboratories				

Table 2. Comparison of TCE Prenatal Developmental Toxicity Study Methods				
	Dawson et al. (1993)	Johnson et al. (2003)	Fisher et al. (2001)	Carney et al. (2006)
Group sizes are range of actual group size (i.e., no. of dams) on study; numbers in parentheses () indicate target group size.				
a = Whole-body exposure, dynamic air flow, analytical chamber concentrations.				
b = Unanimous agreement of cardiac diagnoses by study investigators: a pathologist, a pediatric cardiologist, and a veterinarian.				
Cardiac evaluation references: Staples exam: (Stuckhardt and Poppe, 1984; Staples, 1974); University of AZ exam: (Johnson et al., 2003; Dawson et al., 1993)				

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